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Human IFN-γ

ELISpot MAX™ Deluxe Set

Cat. No. 430124

BioLegend's ELISpot MAX™ Deluxe Set Human IFN-γ contains the components necessary for the detection of Human IFN-γ secreting cells, allowing single cell analysis. It can be used to measure the frequency of human IFN-γ secreting cells.

It is highly recommended that this instruction sheet be read in its entirety before using this product. Do not use this set beyond the expiration date.

Materials Provided

1. Human IFN-γ ELISpot MAX™ Capture Antibody (200X)
2. Human IFN-γ ELISpot MAX™ Detection Antibody (200X)
3. Human IFN-γ Standard (used as a positive control)
4. Avidin-HRP (1000X)
5. TMB Membrane Substrate
6. Coating Buffer A (5X)
7. Assay Diluent B (5X)

Introduction

Human IFN-γ (Interferon-gamma) is a multifunctional cytokine which is the sole member of the class II Interferon. Although a variety of immune cells can express IFN-γ, most of IFN-γ is produced by T-cells and NK cells. It has critical roles in many processes including but not limited to anti-viral response, regulating Th1/Th2 balance, enhancing antigen presentation, and controlling cellular proliferation and apoptosis. IFN-γ can bind to 2 receptors: IFNGR1 and IFNGR2, which transduce the signal downstream through the JAK-STAT pathway. Because of its multifunction nature, dysregulation of IFN-γ expression and activity has been implicated in many diseases such as autoimmune and cancer.

Principle of the Test

BioLegend's ELISpot MAX™ Deluxe Set contains pre-optimized essential reagents and additional buffers and solution for Enzyme-Linked Immunosorbent Spot assay. This kit is designed for detection of human IFN-γ secreting cells. A monoclonal antibody specific for human IFN-γ is coated on ethanol-activated 96-well PVDF membrane plate as the capture. Stimulated and/or unstimulated cells are pipetted directly into the wells. The plate is then placed into a 37°C cell incubator for a period of time. During this incubation period, the capture antibody binds to human IFN-γ from the secreting cells in the vicinity. Then, cells are removed, and a biotinylated anti-human IFN-γ detection antibody is added, followed by avidin-HRP. Subsequently, TMB membrane substrate is added. The TMB membrane substrate reacts with HRP to form a blue-black precipitate at the sites of human IFN-γ. The end result is visible spots, which corresponds to footprints of IFN-γ secreting cells, on the membrane surface.

For research purposes only. Not for use in diagnostic or therapeutic procedures.

Materials to be Provided by the End-User

- PVDF-bottom plate: Millipore MultiScreenHTS IP Filter Plate (Catalog No.) is recommended.
- Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20
- Plate Sealers: BioLegend Cat. No. 423601 is recommended
- 0.22 µm filter
- 35% Ethanol
- Sterile PBS (Phosphate-Buffered Saline): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 µm filtered
- Sterile cell culture media
- Biosafety cabinet
- 37°C CO2 incubator
- Deionized (DI) water
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Wash bottle, manifold dispenser, or automated microplate washer
- Tubes
- Timer
- Stereomicroscope or an automated ELISpot reader

Storage Information

- Store kit components between 2°C and 8°C.
- After reconstitution of the lyophilized standard, which serves as positive control, with the cell culture media, aliquot into polypropylene vials and store at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
- Prior to use, bring all components to room temperature (18°C-25°C). Upon assay completion return all components to appropriate storage

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/support/#msds).
2. TMB Membrane Substrate is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Sample Preparation

This assay has been validated with human peripheral blood mononuclear cells (PBMCs). However, the types of cells, reagents or methods of stimulation, cell separation/sorting protocol, length of incubation are to be determined by the investigator.

If mononuclear cells are to be isolated from whole blood, BioLegend's Lymphopure™ (Catalog No. 426201 or 426202) is the recommended reagent.

It is recommended but not required to have the following controls in each Human IFN-γ ELISpot experiment:

Positive control: use the 1 ng/mL recombinant Human IFN-γ sample prepared from lyophilized standard (See Reagent Preparation section).

Unstimulated cell or negative control: use the same number of cells in cell culture media without any stimulation.

Background control: use sterile cell culture media.

Reagent Preparation

Do not mix reagents from different sets or lots. Reagents and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

NOTE: Precipitation of 5X Assay Diluent B may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the Buffer. If heavy precipitation is observed after the dilution to 1X Assay Diluent B, it can be filtered to clarify the solution.

Preparation of 1X Reagent for 1 Plate

Material	Dilute with
2.4 mL of Coating Buffer A (5X)	9.6 mL of Deionized Water
60 µL of Capture Antibody (200X)	12 mL of 1X Sterile Coating Buffer A
12 mL of Assay Diluent B (5X)	48 mL of PBS
60 µL of Detection Antibody (200X)	12 mL of 1X Assay Diluent B
12 µL of Avidin-HRP (1,000X)	12 mL of 1X Assay Diluent B

Sterile Coating Buffer A: After diluting Coating Buffer A to 1X in deionized water, filter-sterilize with a 0.22 µm filter inside a biosafety cabinet.

Cell Culture Media: For blocking the plate and incubating cell, we recommend cell culture media containing 10% fetal calf serum (FCS).

Positive Control: Lyophilized vials are under vacuum pressure. Reconstitute the Human IFN-γ Standard with 1 mL of the same cell culture media that is used to incubate cells (Refer to ELISpot MAX Kit Lot-Specific Certificate of Analysis/ELISpot MAX Kit Protocol). Allow the reconstituted standard to sit for 15 minutes at room temperature, then mix gently prior to making dilutions. Then, prepare 500 µL of the Positive Control at a concentration of 0.5 ng/mL from the stock solution using the appropriate cell culture medium.

Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

A. Coating (Sterile condition)

1. Dilute Capture Antibody in 1X Sterile Coating Buffer A as described in Reagent Preparation.
2. Activate the 96-wells PVDF bottom plate: open the plate inside a biosafety cabinet, add 20 µL 35% Ethanol per well, and then incubate at room temperature (RT) for 30 seconds.
3. To remove residual Ethanol, wash plate 4 times with 300 µL **sterile PBS** per well. Invert and gently blot plate against sterile absorbent paper towel. **All subsequent washes should be performed similarly.**
4. Add 100 µL of the diluted Capture Antibody per well. Replace lid and incubate overnight (16-18 hours) between 2°C and 8°C.

B. Cell Incubation (Sterile condition)

5. Wash plate 4 times with **sterile PBS**.
6. Add 200 µL cell culture media to each well to block the plate.
7. Incubate for 30 minutes at room temperature.
8. Wash plate 4 times with **sterile PBS.**
9. Add 100 µL of culture media containing Human IFN-γ secreting cells. Add 100 µL of the positive control, if desired.
10. Put plate into a humidified 37°C CO_2 incubator. Incubation time is determined by the investigator. **Do not move or disturb the plate during the incubation period.**

C. Detection, HRP, and spots development (the following steps can be performed outside the biosafety cabinet, if desired)

11. Discard the cell culture media from the plate.
12. Add 300 µL Wash Buffer per well.
13. Incubate at RT for 5 minutes to remove all cells.
14. Discard the wash buffer from the plate.
15. Wash plate 4 times with **Wash Buffer**.
16. Add 100 µL of diluted Detection Antibody solution to each well.
17. Seal plate and incubate at RT for 1 hour.
18. Wash plate 4 times with **Wash Buffer**.
19. Add 100 µL of diluted Avidin-HRP solution to each well.
20. Seal plate and incubate at RT for 30 minutes.
21. Remove the underdrain of the plate and rinse the backside of the PVDF membrane with deionized water. Then, rinse the underdrain itself with water. Gently blot the plate before replacing the underdrain. This will help minimize background because some reagents might leak through the membrane, into the underdrain.
22. Wash plate 5 times with **Wash Buffer.** For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will also help minimize background.
23. Add 100 µL of TMB Membrane Substrate and incubate **in the dark** while monitoring spots development occasionally**.** until distinct blue-black spots appear (roughly 15 minutes for human PBMCs). The membrane of positive control wells should turn blue-black. It is not necessary to seal the plate during this step.
24. Stop reaction by decanting the substrate from the plate, remove the underdrain again, and then washing the plate thoroughly with deionized water.
25. Leave plate to dry at Room Temperature in a clean dark place overnight. Plate should be completely dry before analysis.
26. Count spots via a stereomicroscope or an automated ELISpot reader.

***Optimal substrate incubation time depends on laboratory conditions, cell types, cell donors, stimuli, and stimulation dosage.**

Performance Characteristics

Specificity: the specficity of the antibody pairs used in this ELISpot kit is validated in LEGEND MAX™ Human IFN-γ ELISA kit developed at BioLegend (Catalog No. 430107).

Troubleshooting

The number of spots is lower than expected eventhough the positive control wells turn blue-black.

- Cells are not responding to the stimuli
- Too few cells are added per well

The number of spots is too high for quantification

- Too many cells are added per well

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